Brain Pathology 10: 395-401(2000)

RESEARCH ARTICLE

Chromosomal Imbalances in Brain Metastases of Solid Tumors

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Metastases account for approximately 50% of the malignant tumors in the brain. In order to identify structural alterations that are associated with tumor dissemination into the central nervous system we used Comparative Genomic Hybridization (CGH) to investigate 42 brain metastases and 3 primary tumors of 40 patients. The metastases originated from lung cancer (14 cases), melanomas (7), carcinomas of breast (5), colon (5), kidney (5), adrenal gland (1) and thyroid (1). In addition, tumors of initially unknown primaries were assessed in 3 cases. The highest incidence of DNA gains were observed for the chromosomal regions 1q23, 8q24, 17q24-q25, 20q13 (>80% of cases) followed by the gain on 7p12 (77%). DNA losses were slightly less frequent with 4q22, 4q26, 5q21, 9p21 being affected in at least 70% of the cases followed by deletions at 17p12, 4q32-q34, 10q21, 10q23-q24 and 18q21-q22 in 67.5% of cases. Two unusual narrow regional peaks were observed for the gain on 17q24-q25 and loss on 17p12. The incidence at individual loci can be viewed at our CGH online tumor database at http://amba.charite.de/cgh/. The metastases of each tumor type showed a recurrent pattern of changes. In those cases with primary tumor and metastases available, the CGH pattern exhibited a high degree of conformity. In conclusion, our data suggests that specific genetic lesions are associated with tumor dissemination into the nervous system and that CGH analysis may be a useful supplementary tool for classification of metastases with unknown origin.

Introduction

Brain metastases constitute a major complication of neoplasia. By far the most frequent sites of the primary tumors are lung carcinomas in approximately 50% of cases followed by breast cancer, melanomas, carcinomas of the kidney and the colorectum (18). In 11% of patients the site of the primary lesion is unknown. Although histology and immunohistochemistry are powerful tools in the identification of these primaries a small percentage of cases remain unsolved.

Little is known about the biology of brain metastasis formation. Recently, a brain metastasis-associated ganglioside has been identified (5). Expression of the p21 (WAF1/Cip1) gene has been reported to be decreased in brain metastases of colon carcinomas compared to primary tumors. However, metastatic breast carcinomas showed a higher expression than primaries (24). Similarly the genetic mechanisms that determine the potential of certain tumors to develop brain metastases are largely unknown. Decreased expression of the nm23 gene and loss of heterozygosity on chromosome 15q and 21q11.1-q21.1 have been reported (6, 10, 27). A cytogenetic study on brain metastases of malignant melanoma revealed preferential aberrations on chromosome 11q and isochromosome 17q formation (15, 16) and amplification of the EGF receptor has been found in three out of 18 metastases (26).

Our study was designed to address the questions whether there are genetic alterations that are associated with metastasis formation in the brain. Knowledge of such characteristic features may assist in classifying metastases by their genetic alterations thus contributing to the identification of unknown primary tumors.

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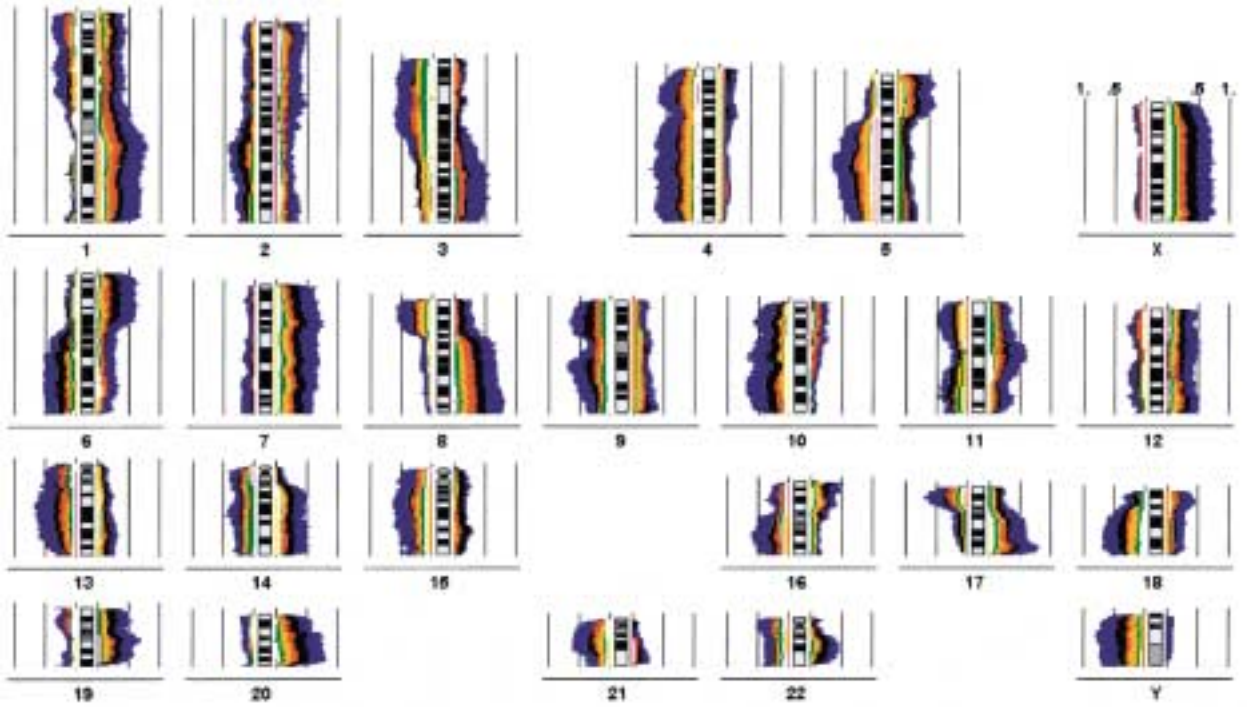

Figure 1. Super histogram of the chromosomal imbalances in 40 brain metastases. DNA losses are indicated by the incidence curve on the left side of the chromosome ideogram, DNA gains are shown on the right side. The .5 and 1. vertical lines represent the 50% and 100% incidences, repectively. Each tumor type is indicated by different colors. *blue*, lung carcinomas (n=14), *black*, melanomas (n=7), *red*, breast cancer (n=5), *orange*, colorectal carcinomas (n=5), *green*, kidney carcinomas (n=4), *white*, unknown primaries (n=3), *yellow*, thyroid carcinoma (n=1), *magenta*, adrenal gland carcinomas (n=1). A peak incidence is seen for the DNA gain on chromosome 17q24-q25.

Materials and methods

Tumor samples. In total, 42 brain metastases and 3 primary tumors of 40 patients were investigated by CGH. The metastases originated from patients with lung cancer (14 cases), melanomas (7), carcinomas of breast (5), colon (5), kidney (4), adrenal gland (1) and thyroid (1). In addition, tumors of initially unknown primaries were assessed in 3 cases. The tumor specimens were mainly obtained during neurosurgical operations at the Charité Hospital and the University Hospitals of Bonn and Poznan. In two cases tumor tissue from autopsies at the Institutes of Pathology of the Charité and the University Hospital of Kiel were investigated in which the primary tumors and two metastases were analyzed. In one additional case the primary tumor of the lung was analyzed from a patient who underwent lung lobectomy at the surgical department of the Charité. DNA was extracted from frozen tumor tissue except for two cases in which only formalin fixed paraffin embedded tissue was available.

Immunohistochemistry and HPV assessment. Immunohistological stains were performed as previously described in the cases of unknown primary carcinomas for the markers cytokeratin 7, cytokeration 20, TTF-1 (Thyroid Transcription Factor 1) and CEA (8). Similarly, the HPV analysis by PCR was done as recently published (7). After CGH analysis, the same DNA sample was used for HPV which was investigated in the majority of cases except for the 2 autoptic lung cancer cases, 2 colon carcinomas, 7 melanomas and the 3 metastases of unknown metastases. In 6 cases the HPV analysis was repeated using DNA from an paraffin block since the initial results were ambiguous.

CGH analysis. The protocols for CGH preparation have been described previously and are available on our web site at http://amba.charite.de/cgh/. Similarly, image acquisition and digital image analysis were performed as described. At least 10 metaphases/karyograms were analyzed per tumor sample with computation of CGH sum-karyograms and mean ratio profiles with confidence intervals (19, 23).

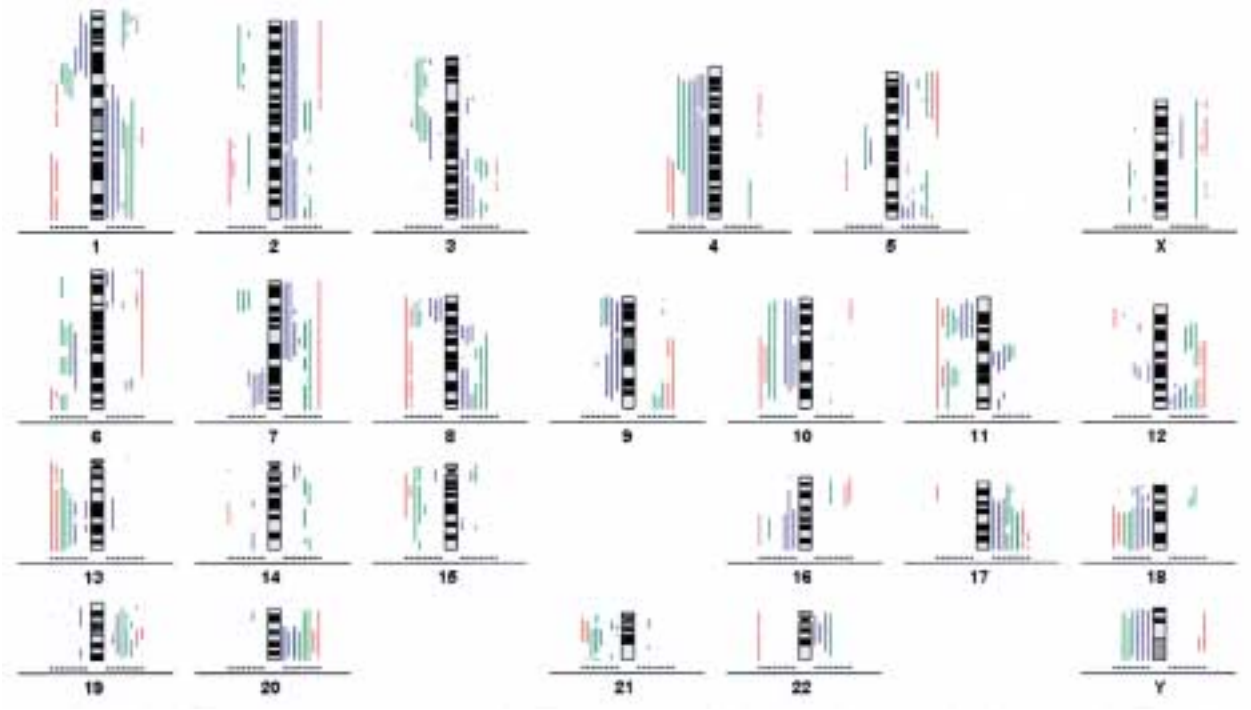

Figure 2. The CGH results of 3 primary tumors and their corresponding brain metastases in a graph representation. Lines on the left side of the chromosome ideogram correspond to deletions, lines on the right to DNA overrepresentations. The imbalances of single tumors can be identified by the indicated position. The tumor samples of the same patient are shown in distinct colors with the primary tumor in the position closest to the ideogram. In case 1 and 2, two metastases were analyzed. The clonal relationship between the primary tumor and their metastases is clearly visible by the high number of common aberrations.

Determination of chromosomal imbalances. DNA gains and losses were determined by statistical methods, i.e. deviations of the mean FITC:TRITC profiles from the normal ratio of 1.0 were tested for significance by a Student's t test (20). In this test we required at least a significance of 95% to score an alteration as a DNA gain or loss. After the statistical determination of the chromosomal imbalances of each tumor sample, a histogram of all cases was calculated (Figure 1). It included one brain metastasis per case and represents the incidence of DNA gains and losses of this tumor collective along each chromosome, showing gains on the right side of the ideogram and losses on the left of the ideogram, e.g. the maximum value of 100% is reached if all samples of the group carry a change at a specific chromosomal region. In the histogram of Figure 1 as well as in the line representation of Figure 2 only alterations with 99% significance in the Student's t test are shown. In addition, we calculated a histogram of all cases including those changes with 95% significance. The histograms and ratio profiles of individual tumors are also available at our web site at http://amba.charite.de/cgh/.

Pronounced DNA gains and losses were defined as those alterations for which the ratio profiles exceeded the values of 1.5 and 0.5, respectively (21). They most likely represent high copy number amplifications or multi copy deletions. Numerical aberrations were determined by an algorithm of our custom made CGH software (23) using the alterations with 99% significance.

Results

Chromosomal imbalances of 40 brain metastases. We observed the highest frequency of changes for the overrepresentations. The DNA gains on chromosome 8q24, 17q24-q25, 20q13 were each present in up to 82.5% of cases followed by the gains on 1q23 and 7p12 with an incidence of 80% and 77.%, respectively. In particular, the band 17q24-q25 showed a peak in the incidence curve compared to the adjacent chromosome regions. Deletions were found less frequently with a maximum of 77.5% on chromosome 4q26 followed by losses on 4q22, 5q21, 9q21 with 70% and deletions at 17p12, 4q32-q34, 10q21, 10q23-q24 and 18q21-q22 in 67.5% of cases. In Figure 1 the chromosomal imbalances of 40 brain metastases are shown in a histogram which represents the DNA gains and losses as an inci-

	pronounced DNA gains[1]	pronounced DNA losses[2]
Breast (n = 5)	1q21-q31 (ii), 1q31-q32 (iii), 1q44 (ii), 3q24-qter (ii), 8q21-qter (iii), 8q23-q24 (iv), 20p12-p13 (ii)	5q21-q22 (ii), 8p12-p23 (ii), 10q24-q25 (ii), 12p11-p12 (ii), 17p12-p13 (ii)
Colon (n = 5)	7q11.2 (ii), 8q13-q21.1 (ii), 8q21.3-qter (iv), 9q34 (ii), 20q11-qter (iii)	4p15.1 (ii), 8p12-pter (iii), 8p21 (iv), 18q12-qter (ii), 18q22 (iii)
Kidney (n = 4)	7q11.2-qter (ii)	
Lung (n = 14)	1q21-q24 (v), 3q23 (v), 3q27-q28 (vii), 5p12-p14 (iv), 7p21-p22 (v), 8q22-qter (iv), 17q25 (v)	4q26 (v), 4q32-q33 (v), 5q21 (iv)
Melanoma (n = 7)	7p15-pter (ii), 7p15-q11.2 (iii), 20p13 (ii), 20q11.2-qter (ii)	

[1]ratio >1.5, [2]ratio <0.5, only those alterations which occurred at least twice were listed with the number indicated in brackets

Table 1. Pronounced DNA imbalances in brain metastases.

dence curve along each chromosome. The same histogram is also available at our CGH online tumor database at http://amba.charite.de/cgh/ where the incidences can be assessed interactively by opening the Infoscreen and pointing at specific chromosomal sites using the computer mouse.

HPV analysis. No genomic sequences of Human Papilloma Viruses were detectable by PCR in any of 24 investigated brain metastases.

Most prevalent changes in single tumor types. The alterations of the tumor groups are visualized by different colors in the histogram (Figure 1). For specific chromosomes, the imbalances of the different entities were partially overlapping. However, the tumors from the same primary site were characterized by a recurrent pattern of aberrations including pronounced DNA gains and losses (Table 1).

Breast cancer metastases had a mean of 39.6 aberrations per case, i.e. 20.2 gains and 19.4 losses. Lung metastases indicated a similarly complex spectrum of aberrations with a mean of 35.6 alterations per tumor with gains and losses almost equally distributed, i.e. 18.7 versus 16.9 thus causing problems particularly in differentiating metastases of these two primary sites. In this regard, the patterns of chromosomes 3 and 12 are important since lung cancer carried deletions on 3pter and very rarely 3p gains whereas breast carcinomas harbored deletions predominantly in the centromeric 3p regions which was often accompanied by gains on 3pter. Chromosome 12p showed deletions in breast cancer in contrast to 12p gains in lung cancer.

Melanomas carried a mean number of 28.6 alterations per case, 16.0 gains and 12.6 losses. Most prevalent changes were gains on chromosome 6p, 7, 8q, 20, 22q, and losses on 6q, 9p and 10q. Pronounced DNA gains were seen most often at 7p15-q11.2 (Table 1).

Colon carcinomas carried a mean of 28.2 aberrations per case, i.e. 15.8 gains versus 12.4 losses. The typical alterations of metastatic colon cancer were deletions on 4p, 4q, 5q21, 8p, 18q and overrepresentations on 8q, 16p12, 20q. In particular, the CGH correlate of an 8q isochromosome with a pronounced deletion on 8p along with a pronounced gain on 8q was most often found in this tumor type. Similarly, 20q amplifications were a frequent finding (Table 1).

Kidney carcinomas harbored more deletions than DNA gains, 13.5 versus 12.3, respectively. The most typical change is the deletion of 3pter, however for differentiation purposes particularly the DNA gain of 5q and the deletions of 14q may be used.

The adrenal gland carcinoma harbored 19 alterations, i.e. 10 gains and 9 losses, and the thyroid carcinoma 27, i.e. 11 gains and 16 losses. Individual ratio profiles of these two tumors, the metastases of unknown primaries and the other cases are available at our online CGH tumor database at http://amba.charite.de/cgh/.

The comparison of primary tumors and their corresponding metastases. The chromosomal alterations of three primary lung carcinomas and 5 brain metastases are shown in Figure 2 as a line representation. In all cases, the clonal relationship between the tumors of individual patients were determined on grounds of the high number of overlapping alterations compared to the different ones. Particularly the biopsied patient represented by the red color showed an increase in size of

deletions on chromosomes 2q, 6q, 8, 10q, 11, 15q, and 18q during tumor progression to the metastatic stage (Figure 2).

Metastases of unknown primaries. We investigated three tumors that were diagnosed by the neuropathologist as metastases of unknown primary tumors. One case showed a papillary growth with large anaplastic tumor cells and the features of signet ring cells. CGH suggested a primary lung carcinoma, i.e. the analysis revealed a very complex aberration pattern with deletions on chromosome 3p along with an overrepresentation of 3q. The CGH findings were corroborated by immunohistochemistry which showed binding of TTF-1 (Thyroid Transcription Factor 1) and cytokeratin 7 in the tumor cells whereas no binding was observed for cytokeratin 20.

A second case had typical features of a colon carcinoma, e.g. deletions of 18q and a pronounced gain on 20q. Histology showing predominantly solid growth with focally tubular and cribriform differentiation, necrosis and little desmoplastic stroma supported the diagnosis. In addition, immunohistochemistry revealing a pronounced expression of CEA and cytokeratin 20 and lack of expression of TTF-1 and cytokeratin 7 were also consistent with colon carcinoma.

A third case showed morphological features that were suggestive of renal cell carcinoma with distinct cytoplasmic borders and clear cell appearance. In contrast, immunohistochemistry showed focal expression of TTF-1 suggesting lung carcinoma. The CGH pattern was inconclusive. The histopathology of the three cases is illustrated at our web site.

Discussion

Genetic markers for tumor dissemination into the nervous system. Metastasis formation is a complex process that involves the inactivation of tumor suppressor genes as well as the activation of proto-oncogenes. Tumor dissemination into brain as an example of organ specific metastasis formation may preferentially involve the acquisition of gain of function mutations, e.g. the expression of specific receptors that might mediate the attachment and infiltration of the circulating tumor cells into the nervous system. Therefore, it is interesting that the CGH analysis revealed distinct overrepresentations as imbalances with the highest incidence. Amplifications point to the activation of proto-oncogenes being the classical representatives of tumor associated genes which act by a gain of function. Although it is more difficult to detect DNA losses, we are convinced that the higher incidence of DNA gains represent no CGH artifact since we used a quite sensitive method for the determination of chromosomal imbalances. In addition, we already investigated other tumor collectives in which we identified deletions as the most prevalent aberration type, e.g. primary small cell lung cancer as a tumor with early and widespread tumor dissemination carried more deletions on chromosomes 3p and 10q than any of the overrepresentations in brain metastases (19).

The CGH results indicated that gain of chromosome 17q24-q25 along with those on 8q24 and 20q13 followed by the 1q23 and 7p12 overrepresentations were the most frequent DNA imbalances. We feel that it might be of particular importance for tumor dissemination into the brain for several reasons. First, isochromosome 17q formation has been described in brain metastases of malignant melanoma (15, 16). Second, the 17q24-q25 overrepresentation showed a relatively narrow peak in the incidence curve compared to the gains on 1q23, 8q24 and 20q13. Second, we did not detect this change at a similar frequency in metastases of other organ sites (21, 25).

Additional indirect evidence for a possible association between chromosome 17q gain and brain metastases formation came from recent clinical studies on c-erbB2 positive breast carcinomas using the Herceptin anti-HER2/NEU antibody. One complication of those cancers which respond to the therapy by the antibody is the development of brain metastases (G. Schaller, personal communication). Overexpression of the HER2/NEU proto-oncogene is associated with the overrepresentation of the gene on chromosome 17q21. This might be accomplished by numerical gain of the entire chromosome, of the long arm 17q or by the amplification of a chromosomal subregion. Likely the initial amplicon may include the distal chromosome bands 17q24-q25. Thus it is tempting to hypothesize that the treatment selects for tumor cells that carry only the distal amplicon at 17q24-q25 while suppressing those with the 17q21 gain. The observation that overrepresentations of entire chromosomal arms might be condensed to smaller DNA amplicons during tumor progression is consistent with this hypothesis (21).

Putative candidate gene for the 17q24-q25 regions are the RAS-related RAC3 gene since it belongs to RHO gene family which has already been implicated in metastasis formation (4, 13). Since it is involved in signal transduction the serine/threonine kinase CSNK1D (casein kinase I delta) is also an interesting candidate (11) similar to the interleukin enhancer-binding factor (12).

Candidate gene for the 8q24 amplicon is c-myc

which has already been implicated in tumor progression (2). Also the 20q13 amplification has been already detected in advanced breast carcinomas and has led to the identification of the AIB1 candidate gene (1). Recently we found that the gain on 1q21-q25 is a typical finding in metastatic squamous cell carcinomas of the lung (21). It has already been associated with metastasis formation of kidney carcinomas (3). The DNA gains at 7p12 probably correspond to overexpression and amplification of EGFR gene at this locus (26).

The incidence of deletions at 15q15-q21 (62.5%) and 21q21 (60%) are fairly high and thus consistent with the findings of the allelotype studies in disseminated tumors including brain metastases (10, 27). From the frequency of the DNA losses, however, the loci on 4q, 5q, 9q, 17p, 10q and 18q seems to be more important. Interestingly, NM34-H5, a new homologue of the NM23 gene has been mapped to 5q23-q31 and constitutes a candidate for a putative tumor suppressor gene on this chromosome arm (17).

Differential diagnosis of brain metastases. Histology and immunohistochemistry are the two major methods in tumor classification. Histology with its large variety of conventional staining protocols already provides a pattern of morphological features that has proven to be specific for several tumor types. In addition, immunohistochemistry is capable to detect lineage specific marker proteins thereby supplementing the morphological tumor diagnosis. Markers for the differential diagnosis of solid tumors are in particular the cytokeratins which show a distinct expression pattern in different carcinoma types (14) as well as proteins with rather specific expression in certain tissue and tumor types, e.g. HMB45 in melanoma, Thyreoglobulin in the thyroid, estrogen receptor in breast and the recently identified TTF-1 in lung carcinomas and thyroid cancer (8).

Although both techniques are well established and reliable tools in tumor diagnosis they have certain limitations. Some tumors still do escape a final diagnosis. Most importantly, the detection of protein expression gives only limited insight on the genetic status of a tumor. It does not answer the questions whether two tumors are clonally related or what are the genetic mutations that has led to the specific protein expression.

Genetic analysis is already becoming a supplement for tumor classification. The detection of viral sequences should be particularly helpful in those cases in which two conditions are fulfilled. First, the viral infection is causally associated with tumor formation of the primary tumor and second the viral genome is integrated into the tumor DNA. In this regard, hepatitis B virus and human papilloma virus are putative markers for liver cancer and cervical carcinomas, respectively. Due to its high prevalence, HPV might be particular useful marker for uterine cervix cancer (7). The fact that we failed to detect HPV sequence in brain metastases of other primary sites is consistent with this view. However, additional studies are necessary to investigate the sensitivity and specificity of HPV as a marker for disseminated cervix carcinoma.

In this study we demonstrate that CGH is capable to establish a clonal relationship between the tumors of the same patient. It may thus be used to prove that a metastasis is derived from a putative primary tumor that has been operated on prior to metastasis formation. Although other genetic markers like mutations of the TP53 gene might be used for this purpose (22), a single mutation might not be sufficient to establish a clonal relationship. CGH has the advantage that it gives an overview about the multiple chromosomal imbalances that are the hallmark of almost all solid tumors (9). Therefore, CGH is suitable for answering such questions.

We demonstrate that the recurrent aberration pattern of different tumor types may be used as a supplement for the identification of unknown primary tumors. Additional studies will have to show how reliable CGH is for this purpose compared to other methods. Since many rare tumors metastasizing to the nervous system have not yet been investigated, and the number of brain metastases examined for each tumor type is low, there still remains open questions. However, our study indicates that chromosomal imbalances detected by CGH may be divided into lesions associated with a morphological phenotype and lesions promoting tumor dissemination into the brain.

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